Phylogeny of Opisthokonta and the Evolution of Multicellularity and Complexity in Fungi and Metazoa

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Abstract

While early eukaryotic life must have been unicellular, multicellular life forms evolved multiple times from protistan ancestors in diverse eukaryotic lineages. The origins of multicellularity are of special interest because they require evolutionary transitions towards increased levels of complexity. We have generated new sequence data from the nuclear large subunit ribosomal DNA gene (LSU rDNA) and the SSU rDNA gene of several unicellular opisthokont protists - a nucleariid amoeba (*Nuclearia simplex*) and four choanoflagellates (*Codosiga gracilis, Choanoeca perplexa, Proterospongia choanojuncta* and *Stephanoeca diplocostata*) to provide the basis for re-examining relationships among several unicellular lineages and their multicellular relatives (animals and fungi). Our data indicate that 1) choanoflagellates are a monophyletic rather than a paraphyletic assemblage that independently gave rise to animals and fungi as suggested by some authors, and 2) the nucleariid filose amoebae are the likely sister group to Fungi. We also review published information regarding the origin of multicellularity in the opisthokonts.

Key words: multicellularity, Opisthokonta, Choanoflagellata, Mesomycetozoea, Metazoa, Fungi, *Nuclearia*

Introduction

Transitions from unicellular to multicellular organisms surely are key events in the evolution of life on earth, permitting divisions of labor that have led to sequential increases in developmental complexity. Molecular trees show that the transition of unicellular eukaryotes to multicellularity occurred multiple times in distinct lineages. These analyses also provide a framework for identifying and timing the evolutionary pathways and innovations that led to the origin of multicellular lineages and the subsequent generation of developmental complexity (Wainwright et al, 1993; Cavalier-Smith, 1998). Genomic techniques, increasingly, are clarifying the molecular bases of regulatory gene networks responsible for the cell differentiation and patterning, and that underlie increases in complexity. However, the genomic approach is inherently comparative, and only in combination with phylogenetics will it reach its full potential. To help provide a more robust phylogeny for the origins of multicellularity in the Opisthokonta, which includes the Metazoa, Fungi, Choanoflagellata, and Mesomycetozoea, we conducted a phylogenetic analysis that includes new nuclear ribosomal gene sequences from a nucleariid amoeba and four species of choanoflagellates.

Opisthokont diversity

A variety of phylogenetic analyses based upon comparisons of different gene families show that fungi are specifically related to animals to the exclusion of green plants, alveolates, stramenopiles, and many separate protist lineages (Wainwright et al, 1993; Baldauf and Palmer, 1993). Furthermore, several protistan lineages may have diverged near the branch between animals and fungi. Most of these hypothesized relationships are based on nuclear small subunit ribosomal DNA (SSU rDNA) (Ragan et al 1996; Amaral Zettler et al, 2001; Atkins et al, 2001),

but some are also based on protein coding sequences (King and Carroll, 2001; Snell et al, 2001, Lang et al, 2002) (see Discussion). The clade that comprises metazoan, fungal and protistan taxa, and that is exclusive of other multicellular taxa (such as plants, and red and brown algae), is now known as Opisthokonta (Cavalier-Smith, 1987, 1998). Two synapomorphies present in most of the opisthokont taxa are the presence of flat mitochondrial cristae and a posteriorly uniflagellate motile state (Cavalier-Smith, 1987, 1998).

Despite the common ancestry suggested by molecular data, the affinity of animals with a particular opistokhont clade has been elusive. Many distinct opistokhont lineages vary widely in life history, external morphology and ultrastructural characters. Whithin the Opisthokontha, the Fungi are the most diverse showing non-flagellate and flagellate forms (sometimes with zoospores that can move like amoebae [Sparrow, 1960]), as well as unicellular and multicellular morphologies. The other key lineages within the Opisthokonta are the Mesomycetozoea (Mendoza et al, 2001), also known as the "DRIPs" (Ragan et al., 1996), Icthyosporea (Cavalier-Smith, 1998), and Mesomycetozoa (Herr et al, 1999). These are a group of mostly parasitic protists that infect diverse tissues in several animal hosts, including humans (reviewed in Mendoza et al 2002). The Choanoflagellata are cosmopolitan free-living uniflagellate organisms that that may be either solitary or colonial and they share many characters with sponges (see Nielsen, 2001), and, by extension, to Metazoa. *Corallochytrium limacisporum*, a free-living but non-flagellate protist that inhabits tropical reef waters, resembles thrausochytrids (Labyrinthulomycota, Stramenopila), but is more closely related to choanoflagellates than to either Stramenopila, Fungi or Metazoa (Cavalier-Smith and Allsopp, 1996). *Ministeria vibrans*, another non-flagellate protist, falls within the Opisthokonta in phylogenetic trees based on SSU data (Cavalier-Smith, 2000). Most recently, Amaral Zettler et al. (2001) proposed that free-living

filose nucleariid amoebae are members of Opisthokonta. These authors suggested that the members of this genus, *Nuclearia*, were not likely monophyletic, because a unicellular eukaryotic snail symbiont had been placed, provisionally, in the genus. However, this organism recently was reassigned to a new genus as *Capsaspora owczarzaki* (Hertel et al, 2002), and has been shown to be a member of the Mesomycetozoea by the use of longer ribosomal sequences (Hertel et al, 2002).

Molecular markers

Because morphological synapomorphies that unite the different unicellular and multicellular opisthokont lineages are difficult to identify, molecular characters, particularly SSU sequences and a growing dataset of full-length large subunit ribosomal DNA (LSU) sequences, are used to infer opisthokont relationships. While SSU sequences have not provided sufficient resolution for the deeper divergences within Opisthokonta, the taxon sampling is by far the best available. Here we present new SSU evidence for four choanoflagellates (*Codosiga gracilis, Choanoeca perplexa, Proterospongia choanojuncta* and *Stephanoeca diplocostata*) and new LSU data for a nucleariid amoeba to test two different hypotheses concerning relationships between unicellular opisthokont protists and their multicellular counterparts: 1) Fungi and animals arose independently from two different choanoflagellate lineages rendering this taxonomic group paraphyletic (Cavalier-Smith, 1987). Only a few choanoflagellate sequences were publicly available to rigorously test of this hypothesis. In this paper we challenge this hypothesis with four new sequences from the three different choanoflagellate families: Acanthoecidae, Codonosigidae and Salpingoecidae; (Table 2). 2) Although the SSU data presented by Amaral-Zettler and coworkers (2001) suggested the nucleariid amoebae are opisthokont taxa, their precise phylogenetic analysis left the placement of these taxa unresolved. Here we present LSU

data from *Nuclearia simplex* in an attempt to improve our understanding of this important group of protists.

Materials And Methods

New sequences

We extracted DNA from *Nuclearia simplex* (CCAP (1552/4) using the Puregene Isolation kit according to manufacturer's instructions. We used the primers F63m and 28S-amp developed by Medina et al (2001) to amplify and sequence approximately four kb of the LSU rRNA gene. We performed DNA amplifications by long PCR (94°C:5min -- 94°C:30sec/45°C:1min/65°C:12min) x30 -- 72°C:10min). The enzyme used was a combination of rTth (Perkin Elmer) and vent polymerases (NEB). After A-tailing with Taq polymerase, the PCR product was cloned into a TOPO vector (Invitrogen). A single clone was sequenced in both directions in a LiCor 4200L apparatus (LiCor, Lincoln, Nebraska). For the combined analysis, we obtained the remaining SSU and LSU sequences from GenBank (Table 1). Choanoflagellate genomic DNA was isolated from frozen cell (-80°) samples of four species (Table 1) by pulverizing it in the reagent DNAzol (Chomczynski et al., 1997), followed by centrifugation and ethanol precipitation. We amplified the complete sequences for SSU using eukaryotic-specific primers (Medlin et al., 1988) via PCR (94°C: 2min -- 94°C:10sec/38-48°C:1min/72°C:3min) x30). PCR products were directly sequenced in both directions with an ABI Prism 377 DNA Sequencer (PerkinElmer Instruments, Norwalk, Connecticut).

Phylogenetic analyses

Alignments for both molecules were refined by eye using a multiple sequence alignment editor. We encoded secondary structure in the alignment, identifying stems, loops and bulges,

and manually excluded regions of ambiguous alignment from the final dataset. The final alignment of both genes used in the combined analyses includes 3872 characters, 2317 from the LSU and 1555 SSU alignments respectively. The final SSU alignment includes 1674 characters.

We performed nested likelihood ratio tests (LRT), using Modeltest version 3.0 (Posada and Crandall, 1998), in order to determine the model of sequence evolution for which the data were most likely. The LRT implemented in Modeltest 3.0 indicated that the model that best fit the combined dataset was a TrN+I+Γ (Tamura-Nei + invariants + gamma). The assumed proportion of invariable sites was 0.3369, and the shape parameter (alpha) was 0.5681. The best model chosen by Modeltest for the SSU dataset was GTR+I+Γ (general-time reversible + invariants + gamma). The assumed proportion of invariable sites was 0.2942, and the shape parameter (alpha) was 0.6026.

We used PAUP* 4.0 (Swofford, 2000) for most phylogenetic analyses. We conducted maximum likelihood (ML), minimum evolution (ME), and maximum parsimony (MP) searches. For ME and MP, we performed heuristic searches, with 1000 replicates of random stepwise addition and TBR branch swapping. For ML, we did heuristic searches, with 5 replicates of random stepwise addition and TBR branch swapping. To estimate branch support we performed 100 bootstrap pseudoreplicates for ML (only combined data) and 1000 pseudoreplicates for ME and MP.

Additionally, we produced Bayesian phylogenetic inference trees using MrBayes 2.0 (Huelsenbeck and Ronquist, 2001). We performed exploratory Markov Chain Monte Carlo (MCMC) runs, starting with random trees and a GTR+I +Γ (general-time reversible + invariants + gamma) model of evolution. Subsequently, we ran the heated MCMC chain for 500,000 generations, which was sampled every 10 updates. We discarded 10,000 cycles as burn-in

before estimating joint posterior probabilities. We submitted data matrices and resulting trees to the TreeBase database under submission number SN1520.

Results

Relationships among major groups

Figure 1 shows the optimal ML phylogenetic tree using a combined data set of SSU and LSU sequences while figure 2 is a comparable analysis for only the SSU sequences. Both trees show support values for nodes according to several different phylogenetic methods. Overall, our analyses offer strong support for the monophyly of the opisthokonts but at the same time describe independent animal and fungal clades. In both trees, mesomycetozoeans and choanoflagellates form the sister group to animals, but support for this potential relationship is low. The status of *Nuclearia* as the sister group to Fungi is well supported in the combined analysis, while in the SSU analysis the *Nuclearia* taxa are basal to the animal/choanoflagellate/mesomycetozoean group, all of which are sister to Fungi, but support is lacking. The relationship between mesomycetozoeans and choanoflagellates will require further exploration. Our data suggest that these two lineages form a monophyletic group but with weak bootstrap support. Additionally, the placement of *Corallochytrium* and *Ministeria*, remains uncertain, in part because of the lack of an LSU sequence. Our SSU analysis with many more choanoflagellate and mesomycetozoean species also suggests that extanct choanoflagellates are monophyletic.

Relationships within groups

The combined analyses support the monophyly of the Choanoflagellata although this phylogenetic inference did not include an acanthoecid choanoflagellate. Sampling of SSU

sequences from choanoflagellate species is still sparse, but our additional sequences permit some observations about relations within the crown group. *Acanthoeca*, *Diaphanoeca*, and *Stephanoeca* form a robustly supported clade (Fig. 2). We found the SSU sequences of *Proterosponga choanojuncta* and *Choanoeca perplexa* to be identical.

Members of the newly identified Mesomycetozoea protistan group was divided into two distinct monophyletic orders, based on SSU data and life history traits (Mendoza et al, 2002). Our SSU analysis (Fig. 2) also recovers the same two groups with high support.

The monophyly of Fungi is well supported by all methods of assessing support in the combined analysis and by the Bayesian posterior probabilities in the SSU analysis. Both the SSU and combined analyses support the Ascomycota/Basydiomycota sister group relationship, as well as their monophyly (Figure 2). Although our SSU data do not resolve the basal fungal relationships, our results are congruent with other phylogenetic analyses (Nagahama et al, 1995; James et al, 2000), which showed that the earliest diverging fungal clades were lineages of Chytridiomycota and Zygomycota, and that neither group was monophyletic.

The monophyly of Metazoa is also well supported by our analyses and the branching pattern in this part of the trees is in agreement with published ribosomal phylogenies for this group (Collins, 1998; Medina et al, 2002).

Discussion

Choanoflagellate hypothesis

Traditional morphologic based studies recognize three families of Choanoflagellata (Leadbeater, 1983), according to whether the cells are naked (Codonosigidae), covered with a theca (Salpingoecidae) or surrounded by a siliceous lorica (Acanthoecidae). Of the 12 genera in

Acanthoecidae; we have sequences for species in three of them (*Acanthoeca*, *Diaphanoeca*, and *Stephanoeca*). These three species form a robustly supported clade (Fig. 1), suggesting that these species share a lorica because they had a lorica-bearing ancestor. The possession of a theca, however, does not appear to have phylogenetic significance at the level of this analysis. The two thecate species sampled (*Salpingoeca infusionum* and *Choanoeca perplexa*) are in separate clades, and may possess this character through convergence. This result is not surprising, for example, Leadbeater (1983) noted that one species of naked choanoflagellate (the colonial *Proterosponga choanojuncta*) contains a phase in its life cycle that is indistinguishable from that in a theca bearing species (the sedentary unicellular *Choanoeca perplexa*). Our SSU sequences of these two species are identical, indicating that they are either very closely related or they are different stages in the life cycle of a single species, as implied by Leadbeater's observations. Finally, an available SSU sequence, recovered from an environmental sample of picoplankton (Moon-van der Staay et al., 2001), appears to represent a phantom choanoflagellate that is more closely related to *Salpinoeca infusionum* than to any other sampled species. Cavalier-Smith (1987) proposed that two different lineages of choanoflagellates independently gave rise to animals and Fungi. He suggested that a codosigid choanoflagellate gave rise to animals, whereas Fungi could have evolved from a salpingoecid choanoflagellate. This hypothesis is contradicted by our molecular evidence. Codonosigid (e.g. *Monosiga brevicolis*) and salpingoecid (e.g. *Salpingoeca infusionum*) lineages are included in a well-supported clade distinct from either Fungi or animals in both the combined and SSU analyses. Nevertheless, extinct stem group choanoflagellates are not precluded as the ancestors of Metazoa.

Overall opisthokont phylogeny and character evolution

The relations among a constellation of rather disparate groups within the Opisthokonta have not been clarified by the SSU gene. In addition to the nuclear ribosomal genes, nuclear and mitochondrial protein coding genes studied in some opisthokont taxa (King and Carroll, 2001, Snell et al, 2001, Baldauf and Palmer, 1993, Baldauf and Doolittle, 1997, Lang et al, 2002) supported a close phylogenetic relationship between animals and Fungi to the exclusion of plants, although only a few studies included any of the opisthokont protists (King and Carroll, 2001, Snell et al, 2001, Lang et all, 2002). Data from the protein coding genes EF-2, α and β-tubulins, and actin (King and Carroll, 2001) as well as from hsp70 gene (Snell et al, 2001) indicated that choanoflagellates are part of the opisthokont clade, and possibly the sister group to animals. King and Carroll (2001) also reported a receptor tyrosine kinase (RTK), similar to the metazoan RTKs, which are widely involved in developmental pathways. Whether or not other opisthokont protistan lineages also have RTKs is unkown. A comprehensive analysis (Lang et al. 2002) of 11 conserved mitochondrial proteins from many protists including both a choanoflagellate and a mesomycetozoean places these two lineages in a monophyletic assemblage that contains the Metazoa, with the choanoflagellates strongly supported as the sister group to metazoans and the mesomycetozoean as the most basal lineage in this clade. Although our phylogenetic analysis had weak support for some key nodes, overall it is consistent with the mitochondrial data (Lang et al, 2002). Our data therefore add support to the inference that multicellularity evolved twice in the Opisthokonta, once in the animals, and again in the Fungi.

Although the morphological evidence is quite indicative and partly in agreement with the molecular data (this study, and Lang et al, 2002), the two synapomorphies used to designate the Opisthokonta (flat mitochondrial cristae and presence of a posterior flagellum) are labile phylogenetic characters that have reversed to an ancestral state in some lineages. Examples are

the presence of tubular mitochondrial cristae in *Ichthyophonus hoferi* (Ragan et al, 1996) -- a mesemycetozoean -- and discoidal mitochondrial cristae in the nucleariid amoebae (Amaral-Zettler et al, 2001). Additionally, the nucleariids and the icthyophonid mesomycetozoeans do not produce flagellated cells but amoeba-like cells. Thus, at present there is no clear ultrastructural synapomorphy that is conserved throughout the whole clade. The absence of a universally diagnostic morphological synapomorphy of Opisthokonta is not surprising given the great age and diversity of the clade. We likely will have to rely, therefore, on sequence data and biochemical data (e.g. gene networks) to identify appropriate diagnostic characters for this eukaryotic group. For example, the discovery of a gene fusion between dihydrofolate reductase (DHFR) and thymidylate synthase (TS) in representatives of major eukaryotic lineages to the exclusion of the currently sampled opisthokont taxa (animals, fungi and *Corallochytrium limacisporum*) offers additional evidence for their monophyly (Stechmann and Cavalier-Smith, 2002). These genes are also separately translated in eubacteria, a fact that was used to place the eukaryotic root between opisthokonts and the rest of the eukaryotic domain (Stechmann and Cavalier-Smith, 2002, Simpson and Roger, 2002). Although the separate DHFR and TS translation represents an ancestral state and the taxon sampling is still limited, this genomic trait exemplifies the type of information that will become increasingly valuable in recognizing groups that share common ancestry.

Evolution of complexity in Fungi

Within the Fungi, simple linear multicellularity of hyphae occurs in all major clades (see below), but only Ascomycota and Basidomycota display more complex two and three dimensional multicellularity in the form of sexual spore-producing fruiting bodies. In both of these groups, reversals to unicellular life forms have occurred, for example, *Saccharomyces* and

many other related yeasts in the Saccharomycotina (Ascomycota) or *Cryptococcus albidus* and related species in the hymenomycete clade of Basidiomycota (de Hoog et al. 2000, p 130). Yeasts in the Taphrinomycotina of the Ascomycota, *Schizosaccharomyces* sp. and *Pneumocystis carinii*, may represent reversals to unicellular life, given the presence in that clade of *Neolecta* sp. with macroscopic, multicellular sexual structures (Landvik et al., 1993).

The single posterior flagellum and strong swimming motility of Blastocladiales, Monoblepharidales and Neocallimastigales zoospores (Sparrow, 1960), as well as their amoeboid movement, are features shared with animals, choanoflagellates, Mesomycetozoea, and amoebae, providing a strong argument that the last common ancestor of these groups was aquatic and had these attributes. Together, the Chytridiomycota and Zygomycota account for no more than 2% of fungal species, raising the possibility that multicellular fruiting bodies were an advantage that allowed Ascomycota and Basidiomycota to become far more numerous. Within Ascomycota and Basidiomycota, not all clades have multicellular fruiting bodies but the clades that do [Pezizomycotina in Ascomycota (and Taphrinomycotina, but only in *Neolecta* sp.), Hymenomycetes in Basidiomycota] are more speciose than sibling clades lacking multicellular fruiting bodies (Saccharomycotina in Ascomycota, Ustilaginomycetes and Urediniomycetes in Basidiomycota). However, multicellular reproductive structures are large and more likely to be seen by biologists, which probably biases the counting of these organisms, a possibility that could be tested by thorough surveys of biodiversity. In Opisthokonta, the simple, linear multicellularity of hyphae is found in Mesomycetozoea and Fungi, but whether it arose independently in each or was an ancestral character lost in choanoflagellates and Metazoa is unknown. Hyphae of the earliest diverging fungi, Blastocladiales, show differentiation among hyphal segments in the production of mitotic and meiotic sporangia or gametangia, best

documented in *Allomyces macrogynus* (Emerson, 1941). However, the multicellularity that allows for more extensive differentiation of function is reserved for sexual fruiting bodies of the Pezizomycotina clade of Ascomycota or the Hymenomycetes clade of Basidiomycota. Here, simple switches that specify the production of a sporangium instead of a vegetative hypha cannot explain the many different tissues of in each of the major organs of a mushroom, instead complex coordination of development by many gene products must be invoked (Wessels and Meinhardt, 1994; Kamada, 2002).

Evolution of animal complexity

The Choanoflagellata lineage has a cell morphology that is closely similar to those in Porifera. The microvillar-flagellar structure of the choanoflagellate collar is unique among protistan opisthokonts, and does not occur in other flagellate groups (Karpov, 2000). Only among sponge choanocytes are similar collar cells found. This shared morphological feature suggests a close relationship between choanoflagellates and sponges (e. g. James-Clark, 1868; Fjerdingstad, 1961; Laval, 1971; Leadbeater, 1985). Other metazoan cells may have collar-like structures but they do not closely resemble choanoflagellate cells. The collars are used similarly in feeding in both choanoflagellates and sponges, and hence there are functional as well as morphological resemblances. Flagellar activity creates a current flowing away from the cell apex, thus drawing water and suspended food items to the collar, where particles can be trapped and ingested. The homology of the choanoflagellate cell with sponge choanocytes is compatible with the ribosomal DNA phylogenies and strongly agrees with the mitochondrial data (Lang et al, 2002).

In contrast to choanoflagellates, crown mesomycetozoeans make poor models for metazoan ancestors. If mesomycetozoeans are more basal within Opisthokonta than are choanoflagellates,

then stem mesomycetozoeans likely included free-living, flagellated forms from which both their crown groups and the crown choanoflagellates have descended, following very divergent modes of life.

The adaptive pathway that led from choanoflagellates to the multicellular sponges may have involved the organization of different cell types. Choanoflagellate cells, with only a single microtubule organizing center, cannot bear a flagellum and generate a spindle at the same time (Margulis, 1981). In order to divide, the cells must resorb the flagellum. There are thus two morphological cell phases, one for dividing cells and one for "adult" cells. In colonial choanoflagellates, both cell phases may be present within the colony at the same time and the phases may be patterned, in some cases with dividing cells inside a layer of flagellar cells. Buss (1987) suggested that these cell phases foreshadowed differentiated cell types that evolved within early metazoans.

A plausible pathway for a colonial choanoflagellate to become truly multicellular is for one of the cell phases to become a stem cell, reproducing itself as well as giving rise to the other cell morphology. The morphologically distinct cells are then differentiated cell types. As the morphologies of both phases are encoded within the choanoflagellate genome, the change from cell phase to cell type must involve capturing from environmental or physiologic cues the regulatory signals that mediate the different cytoarchitectures, and embedding them in a developmental repertoire (Valentine, in press). Such a capture is tantamount to establishing the sort of developmental process that is associated with multicellularity. The co-opting of genes and of developmental pathways has clearly occurred between major taxa, such as phyla and classes, by *cis*-regulatory evolution (e. g. Lowe and Wray, 1997 for echinoderms, Akam et al., 1988 for arthropods, and see Carroll et al, 2001; Wilkins, 2002). A variety of detailed

mechanisms could be responsible for such gene recruitment, and at present no evidence indicates what precisely might have happened during the postulated choanoflagellate-sponge transition. In view of subsequent evolutionary events within Metazoa, homeobox genes may be involved.

Conclusions

Multicellularity might be an adaptive strategy for increased body size (e. g. Cox & Bonner, 2001). Certainly, multicellularity is homoplastic among many clades, perhaps as many as 24 (Buss, 1987). However, the major multicellular kingdoms belong to a clade that includes Opisthokonta and Chlorobiota, and innovations in molecular mechanisms may have permitted the rise of true multicellularity in complex body plans (see Stiller and Hall 2002). Reductions in size and complexity within multicellular clades are also well known. The unicellular state exemplified by some Fungi that have close multicellular relatives (e.g., *Saccharomyces*) can most easily be interpreted as a secondary simplification. Simplifications, though not completely reverting to a single-celled state, may also account for the simple body plans found in several metazoan groups, usually parasites (e. g. Myxozoa, Orthonecta, Rhombozoa, and the somewhat more complex Nematomorpha).

As new genomes are being completed for diverse members of the opisthokont clade, a comparative approach will be essential to address these questions. Comparative studies of the developmental and physiological processes that lead to multicellularity, as well as those that lead to reduced numbers of cell types, will thus provide insights into the evolution of both complexity and simplicity. Information on gene regulation throughout ontogeny is rapidly accumulating and is being synthesized (see Carroll et al, 2001, Davidson, 2001, and Wilkins, 2002). It will be of great interest to learn what are the unique features that characterize Opisthokonta and that were implicated in the evolution of the numerous complexity increases found within that clade.

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Figure legends

Fig. 1. Opisthokont phylogenetic analysis of SSU plus LSU rDNA. Best tree obtained by ML. The first three values above branches correspond to bootstrap values for ML, MP and ME respectively. The fourth value corresponds to posterior probabilities from the Bayesian analysis (A single value of 100 indicates same support in all analyses). Unicellular organisms are boxed. Evolutionary distance is represented by the scale bar. See Table 1 for complete species name. The *Nuclearia simplex* strain sequenced in this study is actually the same as the strain labeled N. s.WB in Hertel et al (2002) (Correction made by that author as a personal communication). The *N. simplex* strain, CCAP (1552/4), sequenced in this study is the Heidelberg strain.

Fig. 2. Opisthokont phylogenetic analysis of SSU rDNA. Best tree obtained by ML. The first two values above branches correspond to bootstrap values for MP and ME respectively. The third value corresponds to posterior probabilities from the Bayesian analysis (A single value of 100 indicates same support in all analyses). Unicellular organisms are boxed. Evolutionary distance is represented by the scale bar. See Table 2 for complete species names. **Mucor* can grow as a yeast and *Icthyophonus* makes filaments and sporangia.

Table 1. List of species used in the combined analysis,current taxonomic classification and accession numbers. New sequences are presented in bold.

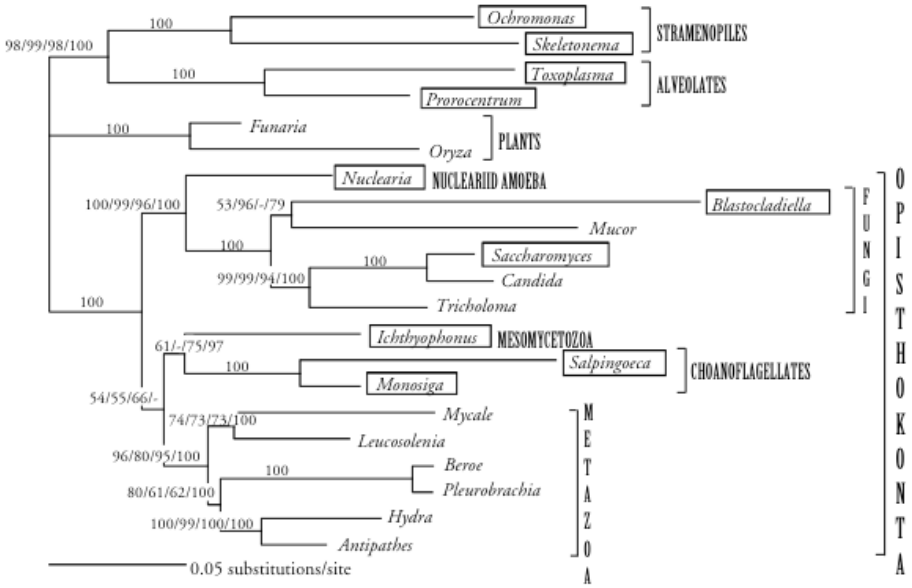
		SSU	LSU
Outgroup taxa			
Oryza sativa	Viridiplantae	X00755	M11585
Funaria hygrometrica	Viridiplantae	X80212	X99331
Prorocentrum micans	Alveolata, Dinophyceae	M14649	X16108
Toxoplasma gondii	Alveolata, Apicomplexa	M97703	X75430
Ochromonas danica	Stramenopile, Chrysophyceae	M32704	Y07977
Skeletonema pseudocostatum	Stramenopile, Bacillariophyta	M54988	Y11512
Opisthokont protists			
Ichthyophonus hoferi	Mesomycetozoean	U25637	AY026370
Monosiga brevicolis ATCC 50154	Choanoflagellida	AF100940	AY026374
Salpingoeca infusionum ATCC 50559	Choanoflagellida	AF100941	AY026380
Nuclearia simplex CCAP (1552/4)	Nucleariidae	AF349566	**AY148095**
Fungi			
Candida albicans	Ascomycota	X53497	X70659
Saccharomyces cerevisiae	Ascomycota	J01355	M27607
Tricholoma matsutake	Basidiomycota	U62538	U62964
Mucor racemosus	Zygomycota	AJ271061	AJ271061
Blastocladiella emersonii	Chytridiomycota	M54937	X90411
Animals			
Mycalefibrexilis	Porifera	AF100946	AY026376
Leucosolenia sp.	Porifera, Calcarea	AF100945	AY026372
Antipathes galapagensis	Cnidaria	AF100943	AY026365
Hydra circumcincta	Cnidaria	AF358080	AY026371
Pleurobrachia bachei	Ctenophora	AF293677	AY026378
Beroe ovata	Ctenophora	AF293694	AY026369

Table 2. List of species,current taxonomic classification and accession numbers for the SSU analysis. * This species was previously placed in the genus *Nuclearia* (see text). New sequences are presented in bold.

		SSU Acces. No.
Outgroup taxa		
Chlorella lobophora	Viridiplantae	X63504
Chlamydomonas reinhardtii	Viridiplantae	M32703
Prorocentrum minimum	Alveolate	Y16238
Toxoplasma gondii	Alveolate	L37415
Ochromonas CCMP584	Stramenopile	U42381
Skeletonema pseudocostatum	Stramenopile	X85393
Choanoflagellates		
Sphaeroeca volvox	Codonosigidae	Z34900
Proterospongia choanojuncta ATCC 50455	Codonosigidae	**AY149896**
Codosiga gracilis ATCC 50454	Codonosigidae	**AY149897**
Monosiga brevicolis ATCC 50154	Codonosigidae	AF100940
Choanoeca perplexa ATCC50453	Salpingoecidae	**AY149898**
Salpingoeca infusorium ATCC 50559	Salpingoecidae	AF100941
Stephanoeca diplocostata ATCC 50456	Acanthoecidae	**AY149899**
Diaphanoeca grandis ATCC 50111	Acanthoecidae	L10824
Acanthoecopsis ungiculata ATCC 50073	Acanthoecidae	L10823
Eukaryote clone OLI11041		AJ402325
Opisthokont inserta sedis		
Corallochytrium limacisporum		L42528
Mesomycetozoea		
Pseudoperkinsus tapetis	Dermocystida	AF192386
Rossette agent of Chinook salmon	Dermocystida	L29455
Dermocystidium salmonis	Dermocystida	U21337
Dermocystidium sp.	Dermocystida	U21336
Rhinosporidium seeberi	Dermocystida	AF118851
Amoebidium parasiticum	Ichthyphonida	AF274751
Amoebidium parasiticum	Ichthyphonida	Y19155
Ichthyophonus hoferi	Ichthyphonida	U25637
Psorospermium haeckelii	Ichthyphonida	U33180
Anurofeca richardsi	Ichthyphonida	AF070445
Sphaeroforma artica	Ichthyphonida	Y16260
**Capsaspora owczarzaki* ATCC 30864		AF349564
Animals		
Leucosolenia sp.	Porifera	AF100945
Suberites ficus	Porifera	AF100947
Rhabdocalyptus dawsoni	Porifera	AF100949

Antiphathes galapagensis	Cnidaria	AF100943
Nanomia bijuga	Cnidaria	AF358071
Chironex fleckeri	Cnidaria	AF358104
Nausithoe rubra	Cnidaria	AF358095
Trichoplax adherens	Placozoa	L10828
Charistephane fugiens	Ctenophora	AF358113
Ptychodera bahamensis	Hemichordata	AF236802
Phoronis hippocrepia	Phoronida	AF202112
Priapulus caudatus	Sipuncula	AF025927
Fungi		
Saccharomyces cerevisiae	Ascomycota	M27607
Neurospora crassa	Ascomycota	X04971
Basidiobulus ranarum	Zygomycota	D29946
Conidiobulus coronatus	Zygomycota	D29947
Piptocephalis corymbifera	Zygomycota	AB016023
Mucor mucedo	Zygomycota	X89434
Glomus mosseae	Zygomycota	Z14007
Monoblepharis insignis	Chytridiomycota	AF164333
Blastocladiella emersonii	Chytridiomycota	M54937
Chytridium confervae	Chytridiomycota	M59758
Coprinus cinereus	Basydiomycota	M92991
Schizophyllum commune	Basydiomycota	X54865
Rhodosporidium toruloides	Basydiomycota	D12806
Nucleariid amoeba		
Nuclearia moebiusi CCAP (1552/3)	Nucleariidae	AF349565
Nuclearia simplex CCAP (1552/4)	Nucleariidae	AF349566
Nuclearia delicatula CCAP (1552/1)	Nucleariidae	AF349563

Figure 1


Ochromonas
Skeletonema
STRAMENOPILES
Toxoplasma
Prorocentrum
ALVEOLATES
Funaria
Oryza
PLANTS
Nucleariа
NUCLEARIID AMOEBA
Blastocladiella
Mucor
Saccharomyces
Candida
Tricholoma
FUNGI
Ichthyophonus
MESOMYCETOZOA
Salpingoeca
Monosiga
CHOANOFLAGELLATES
Mycale
Leucosolenia
Beroe
Pleurobrachia
Hydra
Antipathes
METAZOA
OPISTHOKONTA
98/99/98/100
100
100
100
100
100/99/96/100
53/96/-/79
100
100
99/99/94/100
100
61/-/75/97
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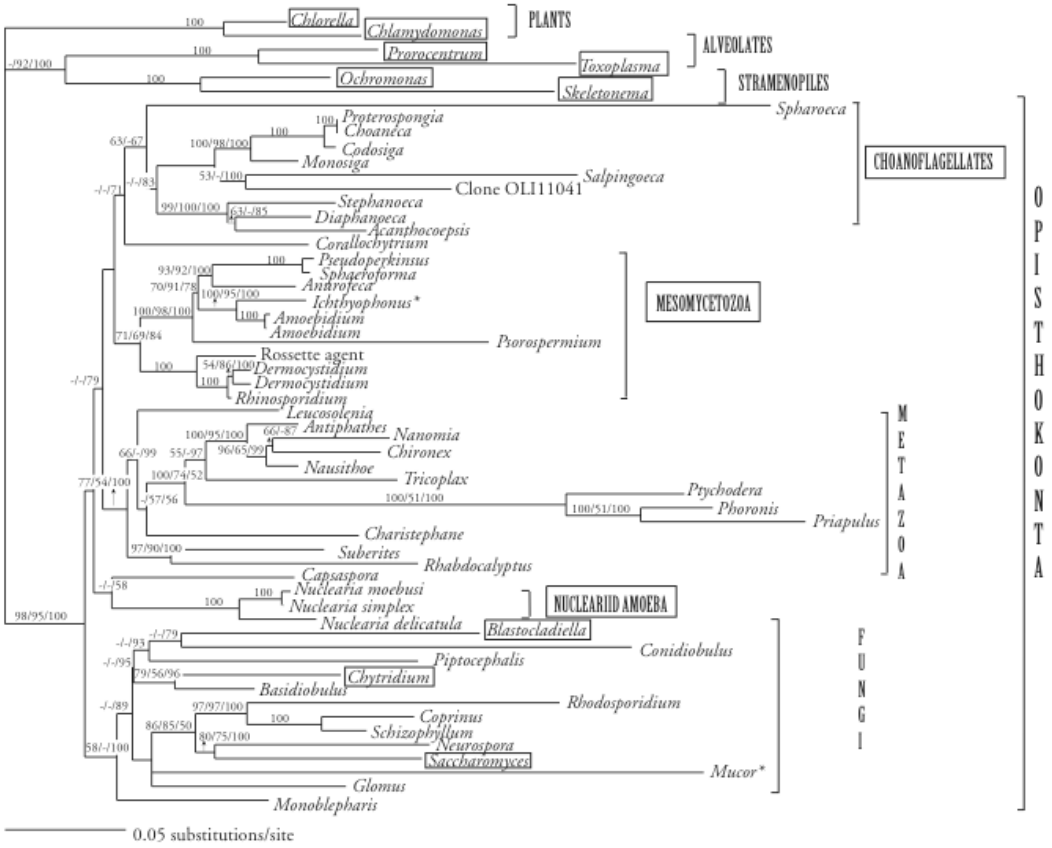
0.05 substitutions/site

Figure 2


Chlorella
Chlamydomonas
PLANTS
Prorocentrum
Toxoplasma
ALVEOLATES
Ochromonas
Skeletonema
STRAMENOPILES
Sphaeroeca
Proterospongia
Choanoeca
Codosiga
Monosiga
Salpingoeca
Clone OLI11041
Stephanoeca
Diaphanoeca
Acanthocoepsis
Corallochytrium
CHOANOFLAGELLATES
Pseudoperkinsus
Sphaeroforma
Amoebidium
Amoebidium
Psorospermium
Rossette agent
Dermocystidium
Dermocystidium
Rhinosporidium
MESOMYCETOZOA
Leucosolenia
Antipathes
Nanomia
Chironex
Nausithoe
Tricoplax
Ptychodera
Phoronis
Priapulus
METAZOA
Charistephane
Suberites
Rhabdocalyptus
Capsaspora
Nucleaira moebusi
Nucleaira simplex
Nucleaira delicatula
NUCLEARIID AMOEBA
Blastocladiella
Conidiobulus
Piptocephalis
Chytridium
Basidiobulus
Rhodosporidium
Coprinus
Schizophyllum
Neurospora
Saccharomyces
Mucor*
Glomus
Monoblepharis
FUNGI
OPISTHOKONTA
0.05 substitutions/site